SureSelect^{XT} Target Enrichment System for Illumina Paired-End Sequencing Library

Illumina HiSeq and MiSeq Multiplexed Sequencing Platforms

Protocol

Version 1.3.1, February 2012

SureSelect platform manufactured with Agilent SurePrint Technology

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Agilent Technologies

Notices

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In this Guide...

This guide describes the recommended operational procedures to capture the genomic regions of interest using the Agilent SureSelect^{XT} Target Enrichment Kit for Illumina Multiplex Sequencing. This protocol is specifically developed and optimized to use biotinylated RNA oligomer libraries to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus, specifically adjusted to provide high performance with SureSelect.

This guide uses an optimized protocol for Illumina paired-end multiplexed library preparation.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the steps to prepare the DNA samples for target enrichment.

3 Hybridization

This chapter describes the steps to prepare and hybridize samples.

4 Addition of Index Tags by Post-Hybridization Amplification

This chapter describes the steps to amplify, purify, and assess quality of the sample libraries. Samples are pooled by mass prior to sequencing.

5 Reference

This chapter contains information on alternative equipment that can be used with this protocol.

What's New in 1.3

- New product configuration and product numbers for SureSelect reagent kits and capture libraries, up to 24 Mb.
- Support for the optional use of the Agilent 2200 TapeStation for DNA quantitation and qualification.
- Support for SureSelect Human All Exon v4 and All Exon v4+UTRs capture libraries.
- Support for Illumina HiSeq and MiSeq platforms.

What's New in 1.2

- Support for large custom captures (up to 34 MB).
- Reagent cap colors are listed where available.
- More details given for the reagent kits to use for each step.
- Update to Covaris shearing volume.
- Update to PCR times in the pre- and post-hybridization library amplification steps.
- Update to cluster generation reagents and procedure.

What's New in 1.1

- Support for the SureSelect Mouse All Exon Kits.

Content

1 Before You Begin 7

Procedural Notes 8
Safety Notes 8
Required Reagents 9
Optional Reagents 12
Required Equipment 13
Optional Equipment 15

2 Sample Preparation 17

Step 1. Shear DNA 20
Step 2. Purify the sample using Agencourt AMPure XP beads 22
Step 3. Assess quality with the 2100 Bioanalyzer 23
Step 4. Repair the ends 25
Step 5. Purify the sample using Agencourt AMPure XP beads 26
Step 6. Add 'A' Bases to the 3' end of the DNA fragments 27
Step 7. Purify the sample using Agencourt AMPure XP beads 28
Step 8. Ligate the indexing-specific paired-end adaptor 29
Step 9. Purify the sample using Agencourt AMPure XP beads 30
Step 10. Amplify adaptor-ligated library 31
Step 11. Purify the sample with Agencourt AMPure XP beads 34
Step 12. Assess quality and quantity with 2100 Bioanalyzer 35

3 Hybridization 37

Step 1. Hybridize the library 40
Step 2. Prepare magnetic beads 46
Step 3. Select hybrid capture with SureSelect 47
Step 4. Purify the sample using Agencourt AMPure XP beads 49

4 Addition of Index Tags by Post-Hybridization Amplification 51

Step 1. Amplify the captured library to add index tags 52
Step 2. Purify the sample using Agencourt AMPure XP beads 56
Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay 57
Step 4. Assess the quantity of each index-tagged library by QPCR 59
Step 5. Pool samples for Multiplexed Sequencing 60
Step 6. Prepare sample for cluster amplification 62

5 Reference 65

SureSelect Reagent Kit Content 66
Other Reagent Kits Content 68
SureSelect^{XT} Indexes for Illumina 70
Alternative Capture Equipment Combinations 71

1 Before You Begin

Procedural Notes 8
Safety Notes 8
Required Reagents 9
Required Equipment 13
Optional Equipment 15

Make sure you have the most current protocol. Go to the SureSelect Related Literature page on genomics.agilent.com and search for manual number G7530-90000 .

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE This protocol differs from the Illumina Multiplexed Paired-End sequencing manual and other SureSelect protocols at several steps. Pay close attention to the primers used for each amplification step and the blocking agents used during hybridization.

NOTE Agilent cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols to process samples for enrichment.

Procedural Notes

- Prolonged exposure of SureSelect Elution Buffer to air can decrease product performance by altering the pH of the solution. Keep the Elution Buffer container tightly sealed when not in use.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing gDNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- Avoid repeated freeze-thaw cycles of stock and diluted gDNA solutions.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
QPCR NGS Library Quantification Kit (Illumina GA)	Agilent p/n G4880A
Herculase II Fusion DNA Polymerase (includes dNTP mix and 5x Buffer) 200 reactions 400 reactions	Agilent p/n 600677 p/n 600679
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
Agencourt AMPure XP Kit 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Life Technologies p/n 4389764
Qubit dsDNA HS Assay Kit *or* Qubit dsDNA BR Assay Kit 100 assays, 2-1000 ng 500 assays, 2-1000 ng 1000 assays, 2-1000 ng	Life Technologies p/n Q32851 Life Technologies p/n Q32850 Life Technologies p/n Q32853 Life Technologies p/n Q33130
Qubit assay tubes	Life Technologies p/n Q32856
Buffer EB (10mM Tris-Cl, ph 8.5)	Qiagen p/n 19086
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

Table 2 Required Reagents for Cluster Generation and Sequencing

Description	Vendor and part number
Illumina Cluster Generation Kit (depending on your instrument and setup)	
TruSeq PE Cluster Kit v5–CS–GA	Illumina p/n PE-203-5001
TruSeq PE Cluster Kit v2–cBot–HS	Illumina p/n PE-401-2001
TruSeq PE Cluster Kit v2.5–cBot–HS	Illumina p/n PE-401-2510
PhiX Control Kit V2 (for HiSeq 2000)	Illumina p/n CT-901-2001
Illumina Sequencing Kit (depending on your instrument and setup)	
TruSeq SBS Kit v5–GA (36-cycle)	Illumina p/n FC-104-5001
TruSeq SBS Kit–HS (50 cycle)	Illumina p/n FC-401-1002

Table 3 SureSelect Reagent Kit

Reagent Kits	16 Reactions	96 Reactions	480 Reactions
SureSelect^{XT} Reagent Kit, HSQ	G9611A	G9611B	G9611C
SureSelect^{XT} Reagent Kit, MSQ	G9612A	G9612B	G9612C

Table 4 SureSelect Capture Library (select one)

Capture Libraries	16 Reactions	96 Reactions	480 Reactions
SureSelect^{XT} Human All Exon 50Mb*	5190-4626	5190-4627	5190-4629
SureSelect^{XT} Human All Exon V4*	5190-4631	5190-4632	5190-4634
SureSelect^{XT} Human All Exon V4+UTRs*	5190-4636	5190-4637	5190-4639
SureSelect^{XT} Mouse All Exon*	5190-4641	5190-4642	5190-4644
SureSelect^{XT} DNA Kinome	5190-4646	5190-4647	5190-4649
SureSelect^{XT} X-chromosome	5190-4651	5190-4652	5190-4653
SureSelect^{XT} Custom 1 kb up to 499 Kb	5190-4806	5190-4807	5190-4809
(reorder)	5190-4811	5190-4812	5190-4814
SureSelect^{XT} Custom 0.5 Mb up to 2.9 Mb	5190-4816	5190-4817	5190-4819
(reorder)	5190-4821	5190-4822	5190-4824
SureSelect^{XT} Custom 3 Mb up to 5.9 Mb	5190-4826	5190-4827	5190-4829
(reorder)	5190-4831	5190-4832	5190-4834
SureSelect^{XT} Custom 6 Mb up to 11.9 Mb	5190-4836	5190-4837	5190-4839
(reorder)	5190-4841	5190-4842	5190-4844
SureSelect^{XT} Custom 12 Mb up to 24 Mb	5190-4896	5190-4897	5190-4899
(reorder)	5190-4901	5190-4902	5190-4904

* For use with SureSelect^{XT} Reagent Kit, HSQ only.

Table 5 Required Reagents for Hybridization

Description	Vendor and part number
Dynabeads MyOne Streptavidin T1	Life Technologies
2 mL	Cat #65601
10 mL	Cat #65602
100 mL	Cat #65603
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

Optional Reagents

Table 6 Optional Reagents

Description	Vendor and part number
SureSelect gDNA Extraction Kit	
50 reaction kit	Agilent p/n G7505A
250 reaction kit	Agilent p/n G7505B
Ethylene glycol	American Bioanalytical p/n AB00455

Required Equipment

Table 7 Required Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
2100 Bioanalyzer	Agilent p/n G2938C
Mx3005P Real-Time PCR System	Agilent p/n 401449 or equivalent
Thermal cycler	Agilent SureCycler, Life Technologies Veriti Thermal Cycler, BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Covaris S-series Single Tube Sample Preparation System, Model S2	Covaris
Covaris microTUBE with AFA fiber and snap cap	Covaris p/n 520045
Microcentrifuge	Eppendorf Microcentrifuge Model 5417C or equivalent
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Qubit Fluorometer	Life Technologies p/n Q32857
Dynal DynaMag-2 magnetic stand	Life Technologies p/n 123-21D or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac or equivalent
Ice bucket	
Powder-free gloves	
PCR tubes, strips, or plates	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Vortex mixer	
Heat block at 37°C	

Table 8 Required Equipment for Hybridization

Description	Vendor and part number
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent
MicroAmp Clear Adhesive Film	Life Technologies p/n 4306311 or equivalent
BD Clay Adams Nutator Mixer	BD Diagnostics p/n 421105 or equivalent
Dynal DynaMag-2 magnetic stand	Life Technologies p/n 123-21D or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Pipet-Light Multichannel Pipette, 12 channels	Rainin p/n L12-20 or equivalent
PCR tubes, strips, or plates	
Sterile, nuclease-free aerosol barrier pipette tips	
Thermal cycler	Agilent SureCycler, Life Technologies Veriti Thermal Cycler, BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Timer	
Vortex mixer	
Water bath or heat block set to 65°C	

Optional Equipment

Table 9 Optional Equipment for Hybridization

Description	Vendor and part number
Tube-strip capping tool	Agilent p/n 410099

Table 10 Optional Equipment for Library Prep and Post-Hybridization Amplification

Description	Vendor and part number
2200 TapeStation System	Agilent p/n G2964AA or G2965AA
D1K ScreenTape	Agilent p/n 5067-5361
D1K Reagents	Agilent p/n 5067-5362
High Sensitivity D1K ScreenTape	Agilent p/n 5067-5363
High Sensitivity D1K Reagents	Agilent p/n 5067-5364

2 Sample Preparation

Step 1. Shear DNA 20
Step 2. Purify the sample using Agencourt AMPure XP beads 22
Step 3. Assess quality with the 2100 Bioanalyzer 23
Step 4. Repair the ends 25
Step 5. Purify the sample using Agencourt AMPure XP beads 26
Step 6. Add 'A' Bases to the 3' end of the DNA fragments 27
Step 7. Purify the sample using Agencourt AMPure XP beads 28
Step 8. Ligate the indexing-specific paired-end adaptor 29
Step 9. Purify the sample using Agencourt AMPure XP beads 30
Step 10. Amplify adaptor-ligated library 31
Step 11. Purify the sample with Agencourt AMPure XP beads 34
Step 12. Assess quality and quantity with 2100 Bioanalyzer 35

This section contains instructions for prepped library production specific to the Illumina paired-read sequencing platform. For each sample to be sequenced, individual library preparations, hybridizations, and captures are performed. The samples are then tagged by PCR with an index (barcode) sequence. Depending on the target size of the SureSelect capture, up to 12 samples can be pooled and sequenced in a single lane using Illumina's multiplex index tags.

The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, and implementation of AMPure XP beads for all purification steps, and primers used for PCR.

Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n 1005361 Rev. C) for more information.

Before you begin, you can use the SureSelect gDNA Extraction Kit to extract genomic DNA. Refer to the *gDNA Extraction Kit Protocol* (p/n 5012-8701).

NOTE Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. Use the Qubit system to quantify genomic DNA before library preparation.

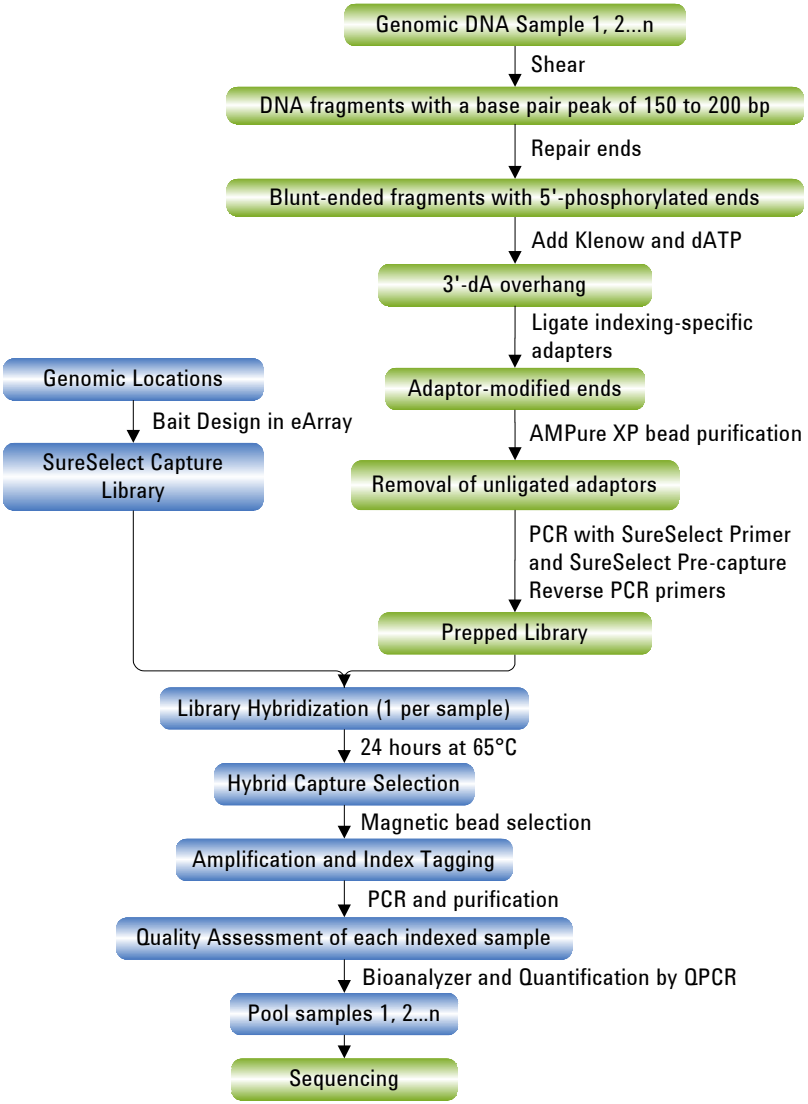

Figure 1 Overall sequencing sample preparation workflow.

Table 11 Overview and time requirements

Step	Time
Illumina Prepped library Production	1 day
Library Hybridization	24 hours (optional 72 hours)
Bead preparation	30 minutes
Capture Selection and Washing	2 hours
DNA purification	30 minutes
Post-Hybridization Amplification	1 hour
PCR purification	30 minutes
Bioanalyzer QC and QPCR	2 to 3 hours
Pool indexed samples by mass	< 1 hour

Step 1. Shear DNA

For each DNA sample to be sequenced, prepare 1 library.

1. Use the Qubit dsDNA Assay to determine the concentration of your gDNA sample. Make sure the gDNA is of high quality (non-degraded, A_{260}/A_{280} is 1.8 to 2.0).

   Follow the instructions for the instrument.

2. Set up the Covaris instrument.
   - **a** Check that the water in the Covaris tank is filled with fresh deionized water to fill line level 12 on the graduated fill line label.
   - **b** Check that the water covers the visible glass part of the tube.
   - **c** Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.
   - **d** *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.
   - **e** On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes before use.

   Refer to the Covaris instrument user guide.

3. Dilute 3 µg of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 130 µL.

4. Put a Covaris microTube into the loading and unloading station.

   Keep the cap on the tube.

5. Use a tapered pipette tip to slowly transfer the 130 µL DNA sample through the pre-split septa.

   Be careful not to introduce a bubble into the bottom of the tube.

6. Secure the microTube in the tube holder and shear the DNA with the settings in Table 12. The target peak for base pair size is 150 to 200 bp.

Table 12 Covaris shear settings

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

7 Put the Covaris microTube back into the loading and unloading station.

8 While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.

9 Transfer the sheared DNA into a new 1.5-mL LoBind tube.

Step 2. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the sheared DNA library (~130 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 50 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove approximately 50 µL of the supernatant to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 3. Assess quality with the 2100 Bioanalyzer

NOTE As an alternative, you can use the D1K ScreenTape (Agilent p/n 5067-5361) and D1K Reagents (Agilent p/n 5067-5362). For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

Use a Bioanalyzer DNA 1000 chip and reagent kit. See the *Agilent DNA 1000 Kit Guide*, at http://www.chem.agilent.com/en-US/Search/Library/_layouts/Agilent/PublicationSummary.aspx?whid=46764.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results.

   Check that the electropherogram shows a distribution with a peak height between 150 to 200 nucleotides.

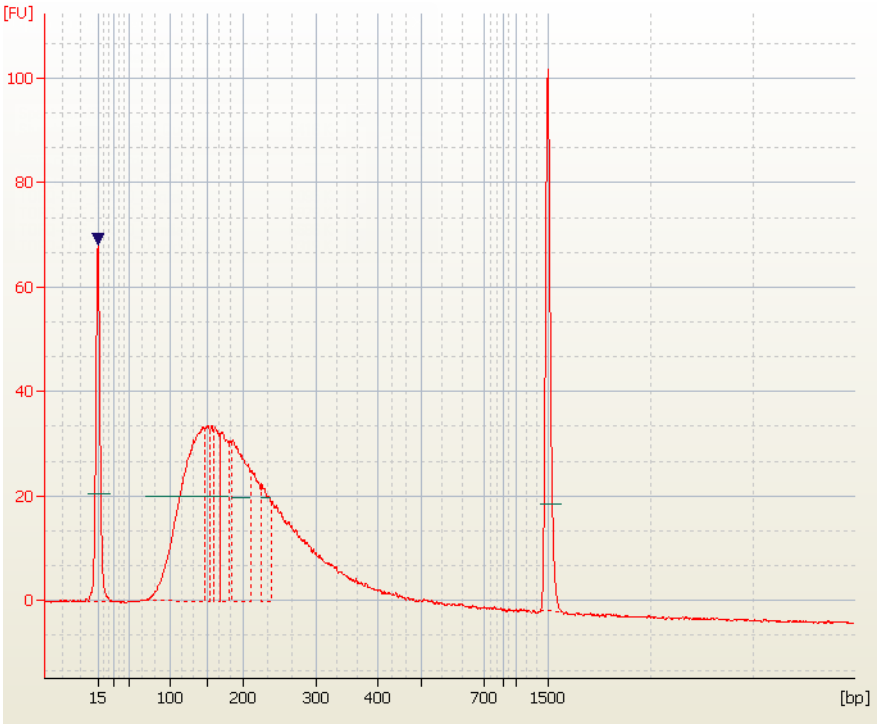

Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay. The electropherogram shows a distribution with a peak size between 150 to 200 nucleotides.

Step 4. Repair the ends

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 12 samples (including excess) are shown in each table as an example.

Use the SureSelect Library Prep Kit, ILM.

Prepare the master mix on ice.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 13.Mix well by gently pipetting up and down.

2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 13.Mix well on a vortex mixer.
   b Add 52 µL of the reaction mix to each well or tube.
   c Add 48 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

3 Incubate in a thermal cycler for 30 minutes at 20°C. Do not use a heated lid.

Table 13 End Repair Mix

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
DNA sample	48 µL	
Nuclease-free water	35.2 µL	440 µL
10X End Repair Buffer (clear cap)	10 µL	125 µL
dNTP Mix (green cap)	1.6 µL	20 µL
T4 DNA Polymerase (purple cap)	1 µL	12.5 µL
Klenow DNA Polymerase (yellow cap)	2 µL	25 µL
T4 Polynucleotide Kinase (orange cap)	2.2 µL	27.5 µL
Total Volume	**100 µL**	**650 µL (52 µL/sample)**

Step 5. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the end-repaired DNA library (~100 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 32 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

Remove the supernatant (~32 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 6. Add 'A' Bases to the 3' end of the DNA fragments

Use the SureSelect Library Prep Kit, ILM.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 14.Mix well by gently pipetting up and down.

2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 14.Mix well on a vortex mixer.
   b Add 20 µL of the reaction mix to each well or tube.
   c Add 30 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 14 Adding "A" Bases

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
DNA sample	30 µL	
Nuclease-free water	11 µL	137.5 µL
10X Klenow Polymerase Buffer (blue cap)	5 µL	62.5 µL
dATP (green cap)	1 µL	12.5 µL
Exo(-) Klenow (red cap)	3 µL	37.5 µL
Total Volume	**50 µL**	**250 µL (20 µL/sample)**

3 Incubate in a thermal cycler for 30 minutes at 37°C.

   If you use a heated lid, make sure that the lid temperature does not exceed 50°C.

Step 7. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the A-tailed DNA library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 15 µL of nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~15 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.
13. Proceed immediately to the next step, "Step 8. Ligate the indexing-specific paired-end adaptor".

Step 8. Ligate the indexing-specific paired-end adaptor

This step uses a 10:1 molar ratio of adaptor to genomic DNA insert, based on a starting quantity of 3 µg of DNA before shearing.

Use the SureSelect Library Prep Kit, ILM.

1 For 1 library (prepare on ice):
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 15.

2 For multiple libraries (prepare on ice):
   a Prepare the reaction mix in Table 15.
   b Add 37 µL of the reaction mix to each well or tube.
   c Add 13 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

Table 15 Ligation master mix*

Reagent	Volume for 1 Library	Volume for 12 Libraries (includes excess)
DNA sample	13 µL	
Nuclease-free Water	15.5 µL	193.75 µL
5X T4 DNA Ligase Buffer	10 µL	125 µL
SureSelect Adaptor Oligo Mix (brown cap)	10 µL	125 µL
T4 DNA Ligase (red cap)	1.5 µL	18.75 µL
Total Volume	**50 µL**	**462.5 µL (37 µL/sample)**

* Included in the SureSelect Library Prep Kit, ILM.

3 Incubate for 15 minutes at 20°C on a thermal cycler. Do not use a heated lid.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 9. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the ligated library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 0.5 mL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 32 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~32 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 10. Amplify adaptor-ligated library

Use reagents from these kits:

- SureSelect Library Prep Kit, ILM
- SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2
- Herculase II Fusion DNA Polymerase (Agilent)

This protocol uses a third of the adaptor-ligated fragments for amplification. The remainder can be saved at 20°C for future use, if needed.

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 For 1 library:
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 16, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   - **a** Prepare the reaction mix in Table 16, on ice. Mix well on a vortex mixer.
   - **b** Add 35 µL of the reaction mix to each well or tube.
   - **c** Add 15 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples.

NOTE The optimal amount of indexing adaptor-ligated library in the PCR is 250 ng as quantified on a bioanalyzer DNA1000 chip.

Table 16 Components for PCR mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Indexing Adaptor-ligated library	15 µL	
Nuclease-free water	21 µL	262.5 µL
SureSelect Primer (brown cap)*	1.25 µL	15.625 µL
SureSelect ILM Indexing Pre Capture PCR Reverse Primer (clear cap)†	1.25 µL	15.625 µL
5X Herculase II Rxn Buffer (clear cap)‡	10 µL	125 µL
100 mM dNTP Mix (green cap)‡	0.5 µL	6.25 µL
Herculase II Fusion DNA Polymerase (red cap)‡	1 µL	12.5 µL
Total	**50 µL**	**437.5 µL (35 µL/reaction)**

* Included in the SureSelect Library Prep Kit, ILM.

† Included in the SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2.

‡ Included in the Herculase II Fusion DNA Polymerase (Agilent) kit. *Do not use the buffer or dNTP mix from any other kit.*

3 Run the program in Table 17 in a thermal cycler.

Table 17

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	30 seconds
Step 3	65°C	30 seconds
Step 4	72°C	1 minute
Step 5		Repeat Step 2 through Step 4 for a total of 4 to 6 times.
Step 6	72°C	10 minutes
Step 7	4°C	Hold

NOTE

Different library preparations can produce slightly different results, based on varying DNA quality. In most cases, 5 cycles will produce an adequate yield for subsequent capture without introducing bias or non-specific products. If yield is too low or non-specific high molecular weight products are observed, adjust the number of cycles accordingly with the remaining extra library template.

As an alternative, you can prepare one PCR master mix as outlined in Table 16. Split the master mix into three small-scale 10 µL PCR reactions and run for 4, 5 or 6 cycles. Clean these PCR reactions using the AMPure XP protocol outlined in "Step 2. Purify the sample using Agencourt AMPure XP beads" with these modifications: Use 30 µL of AMPure XP beads and elute with 20 µL of nuclease-free water. Run these cleaned samples on a DNA1000 chip on the Bioanalyzer, as described in "Step 3. Assess quality with the 2100 Bioanalyzer".

Use the optimal cycle number to repeat PCR at the 50 µL reaction scale.

Step 11. Purify the sample with Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add the amplified library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

Step 12. Assess quality and quantity with 2100 Bioanalyzer

NOTE As an alternative, you can use the D1K ScreenTape (Agilent p/n 5067-5361) and D1K Reagents (Agilent p/n 5067-5362). For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

Use the Bioanalyzer DNA 1000 to assess the quantity, quality and size distribution of the PCR products.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results. Check that the electropherogram shows a distribution with a peak size approximately 250 to 275 bp. Measure the concentration of the library by integrating under the peak.

NOTE A minimum of 500 ng of library is required for hybridization.

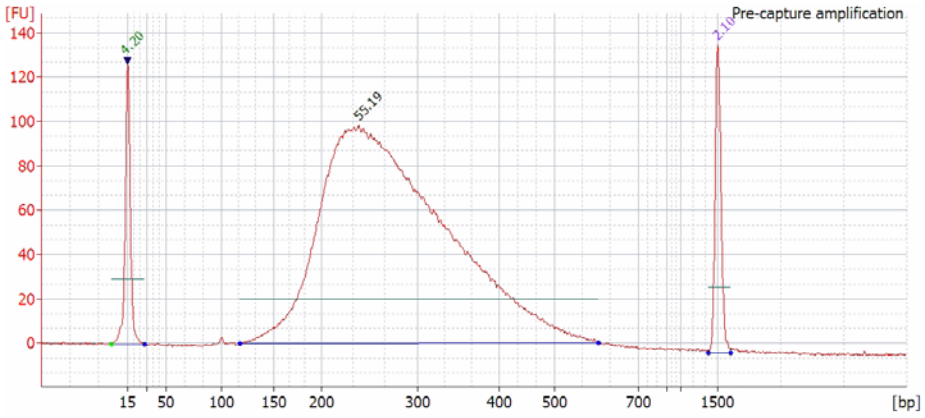

Figure 3 Analysis of amplified prepped library DNA using a DNA 1000 assay. The electropherogram shows a single peak in the size range of 250 to 275 bp.

3 Hybridization

Step 1. Hybridize the library 40
Step 2. Prepare magnetic beads 46
Step 3. Select hybrid capture with SureSelect 47
Step 4. Purify the sample using Agencourt AMPure XP beads 49

This chapter describes the steps to combine the prepped library with the hybridization reagents, blocking agents and the SureSelect capture library. Each DNA library sample must be hybridized and captured individually prior to addition of the indexing tag by PCR.

CAUTION The ratio of SureSelect capture library to prepped library is critical for successful capture.

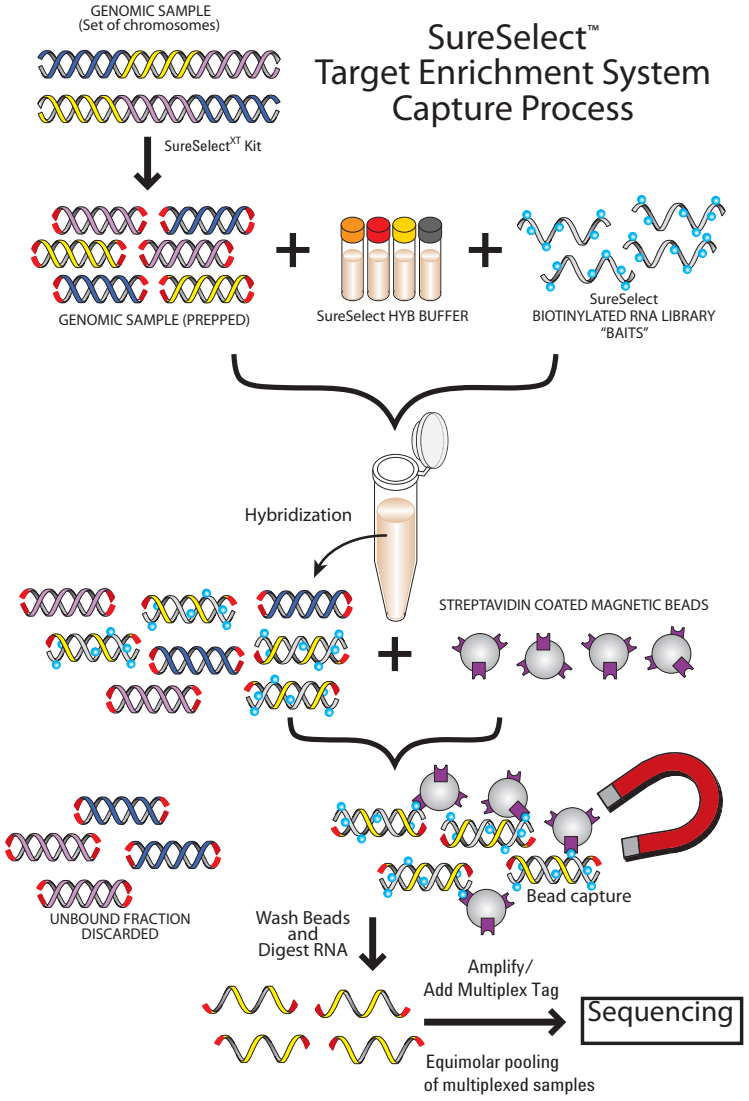

Figure 4 SureSelect Target Enrichment System Capture Process

Refer to "SureSelect Reagent Kit Content" on page 66 for a complete content listing of each SureSelect Target Enrichment kit.

CAUTION

You must avoid evaporation from the small volumes of the capture during the 24 hour or greater incubation.

If you want to use a different combination of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape), first test the conditions. Incubate 27 µL of SureSelect Hybridization Buffer (without DNA) at 65°C for 24 hours (or longer, if applicable) as a test. Include buffer in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 3 to 4 µL.

For a partial list of tested options showing minimal evaporation, refer to "Alternative Capture Equipment Combinations" on page 71.

Step 1. Hybridize the library

For each sample library prepared, do one hybridization and capture. Do not pool samples at this stage.

The hybridization reaction requires 500 ng of DNA with a maximum volume of 3.4 µL.

1. If the prepped library concentration is below 147 ng/µL, use a vacuum concentrator to concentrate the sample at ≤ 45°C.
   a. Add the entire 30 µL of prepped library to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle.

      You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.

   b. Completely lyophilize. Use a vacuum concentrator on low heat (less than 45°C) to dehydrate.

   c. Reconstitute with nuclease-free water to bring the final concentration to 147 ng/µL (or greater if sample recovery is of concern). Pipette up and down along the sides of the tube for optimal recovery.

   d. Mix well on a vortex mixer and spin in a microfuge for 1 minute.
2. *Optional.* To test recovery after lyophilization, reconstitute the sample to greater than 147 ng/µL and check the concentration on a Bioanalyzer DNA 1000 chip. See "Step 12. Assess quality and quantity with 2100 Bioanalyzer" on page 35. After quantitation, adjust the sample to 147 ng/µL.

   Alternatively, concentrate a 500 ng aliquot at ≤ 45°C down to 3.4 µL. If the sample dries up completely, resuspend in 3.4 µL of water and mix on a vortex mixer. If processing multiple samples, adjust to equivalent volumes before concentrating.
3. Mix the components in Table 18 at room temperature to prepare the hybridization buffer.

Table 18 Hybridization Buffer

Reagent	Volume for 1 capture (µL), includes excess	Volume for 6 captures (µL), includes excess	Volume for 12 captures (µL), includes excess
SureSelect Hyb #1 (orange cap, or bottle)	25	125	250
SureSelect Hyb #2 (red cap)	1	5	10
SureSelect Hyb #3 (yellow cap)	10	50	100
SureSelect Hyb #4 (black cap, or bottle)	13	65	130
Total	**49 (40 µL needed)**	**245 (40 µL/sample)**	**490 (40 µL/sample)**

4 If precipitate forms, warm the hybridization buffer at 65°C for 5 minutes.

5 In a PCR plate, prepare the SureSelect capture library mix for target enrichment:

- **a** Keep tubes on ice until step 10.
- **b** For each sample, add the amount of SureSelect capture library as listed in Table 19, based on the Mb target size of your design.
- **c** Use nuclease-free water to prepare a dilution of the SureSelect RNase Block (purple cap) as listed in Table 19.

  Prepare enough RNase Block dilution for all samples, plus excess.
- **d** Add the amount of diluted SureSelect RNase Block (purple cap) listed in Table 19 to each capture library, and mix by pipetting.

Table 19 SureSelect Capture Library.

Capture Size	Volume of SureSelect Library	RNase Block Dilution (Parts RNase block: Parts water)	Volume of RNase Block Dilution to Add
< 3.0 Mb	2 µL	1:9 (10%)	5 µL
≥ 3.0 Mb	5 µL	1:3 (25%)	2 µL

6 Mix the contents in Table 20 to make the correct amount of SureSelect Block mix for the number of samples used.

Table 20 SureSelect Block Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
SureSelect Indexing Block #1 (green cap)	2.5 µL	31.25 µL
SureSelect Block #2 (blue cap)	2.5 µL	31.25 µL
SureSelect Indexing Block #3 (brown cap)	0.6 µL	7.5 µL
Total	**5.6 µL**	**70 µL**

7 In a separate PCR plate, prepare the prepped library for target enrichment.

- **a** Add 3.4 µL of 147 ng/µL prepped library to the "B" row in the PCR plate. Put each sample into a separate well.
- **b** Add 5.6 µL of the SureSelect Block Mix to each well in row B.
- **c** Mix by pipetting up and down.
- **d** Seal the wells of row "B" with caps and put the PCR plate in the thermal cycler. Do not heat the Hybridization Buffer or capture library yet, only the prepped library with blockers.
- **e** Start the thermal cycler program in Table 21.

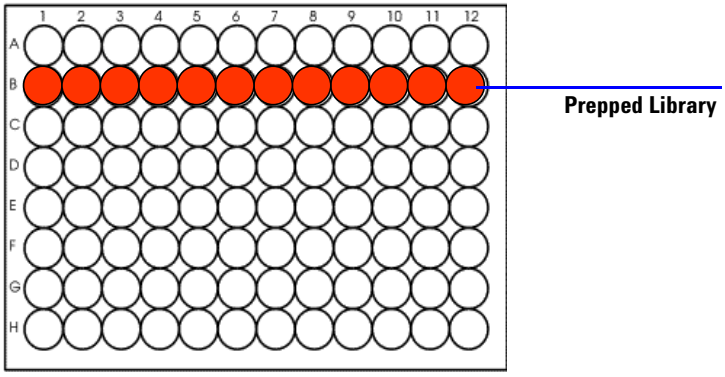

Figure 5 Prepped library shown in red

Table 21 PCR program

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

8 Use a heated lid on the thermal cycler at 105°C to hold the temperature of the plate on the thermal cycler at 65°C.

CAUTION The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

9 Maintain the plate at 65°C while you load 40 µL of hybridization buffer per well into the "A" row of the PCR plate. The number of wells filled in Row A is the number of libraries prepared.

The example in Figure 6 is for 12 captures.

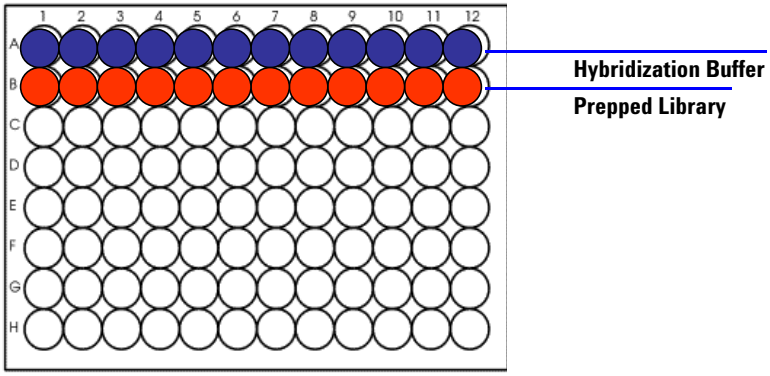

Figure 6 Hybridization buffer shown in blue

Make sure that the plate is at 65°C for a minimum of 5 minutes before you go to step 10.

10 Add the capture library mix from step 5 to the PCR plate:

a Add the capture library mix (7 µL) to the "C" row in the PCR plate.

For multiple samples, use a multi-channel pipette to load the capture library mix into the "C" row in the PCR plate.

Keep the plate at 65°C during this time.

b Seal the wells with strip caps. Use a capping tool to make sure the fit is tight.

c Incubate the samples at 65°C for 2 minutes.

11 Maintain the plate at 65°C while you use a multi-channel pipette to take 13 µL of Hybridization Buffer from the "A" row and add it to the SureSelect capture library mix contained in row "C" of the PCR plate for each sample. (See Figure 7.)

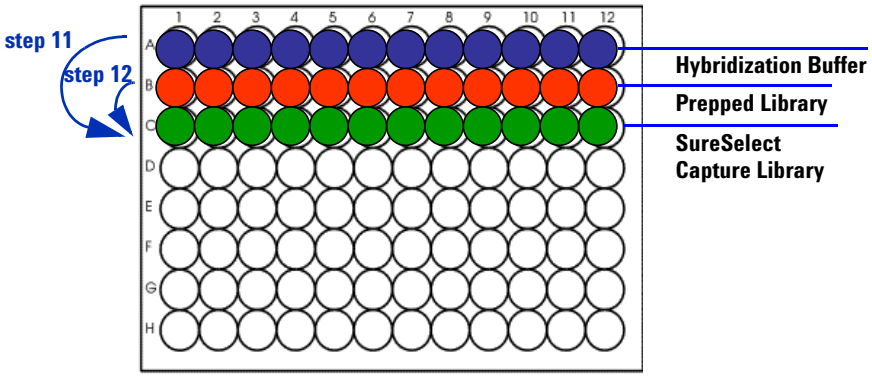

Figure 7 SureSelect Capture Library, or "Baits", shown in Green

12 Maintain the plate at 65°C while you use a multi-channel pipette to transfer the entire contents of each prepped library mix in row "B" to the hybridization solution in row "C". (See Figure 7.) Mix well by slowly pipetting up and down 8 to 10 times.

The hybridization mixture is now 27 to 29 µL, depending on degree of evaporation during the preincubations.

13 Seal the wells with strip caps or double adhesive film. Make sure all wells are completely sealed.

Use new adhesive seals or strip caps. The structural integrity of the seals and caps can be compromised during the previous incubation steps.

CAUTION Wells must be adequately sealed to minimize evaporation, or your results can be negatively impacted.

If you use strip tubes, test for evaporation before you do the first experiment to make sure the tube/capping method is appropriate for the thermal cycler. Check that no more than 3 to 4 µL is lost to evaporation.

14 Incubate the hybridization mixture for 24 hours at 65°C with a heated lid at 105°C.

Step 2. Prepare magnetic beads

Use these reagents from the SureSelect Target Enrichment Kit Box #1:

- SureSelect Binding Buffer
- SureSelect Wash 2

1. Prewarm SureSelect Wash 2 at 65°C in a circulating water bath or heat block for use in "Step 3. Select hybrid capture with SureSelect".
2. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 on a vortex mixer. Magnetic beads settle during storage.
3. For each hybridization, add 50 µL of Dynabeads MyOne Streptavidin T1 to a 1.5-mL microfuge tube.
4. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix the beads on a vortex mixer for 5 seconds.
   c. Put the tubes into a magnetic device, such as the Dynal magnetic separator (Life Technologies).
   d. Remove and discard the supernatant.
   e. Repeat step a through step d for a total of 3 washes.
5. Resuspend the beads in 200 µL of SureSelect Binding Buffer.

Step 3. Select hybrid capture with SureSelect

Use these reagents from the SureSelect Target Enrichment Kit Box #1:

- SureSelect Wash 1
- SureSelect Wash 2
- SureSelect Elution Buffer
- SureSelect Neutralization Buffer

CAUTION Keep the Elution Buffer container tightly sealed when not in use. Prolonged exposure of SureSelect Elution Buffer to air can decrease product performance by altering the pH of the solution.

1. Estimate and record the volume of hybridization that remained after 24 hour incubation.
2. Keep the PCR plate or tubes at 65°C in the PCR machine while you add the hybridization mixture directly from the thermal cycler to the bead solution. Invert the tube to mix 3 to 5 times.

   Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance. See Table 37 on page 71 for tips to minimize evaporation.
3. Incubate the hybrid-capture/bead solution on a Nutator or equivalent for 30 minutes at room temperature.

   Make sure the sample is properly mixing in the tube.
4. Briefly spin in a centrifuge.
5. Separate the beads and buffer on a magnetic separator and remove the supernatant.
6. Resuspend the beads in 500 µL of SureSelect Wash 1 by mixing on a vortex mixer for 5 seconds.
7. Incubate the samples for 15 minutes at room temperature. Occasionally mix on a vortex mixer.
8. Briefly spin in a centrifuge.
9. Separate the beads and buffer on a magnetic separator and remove the supernatant.

10 Wash the beads:

- **a** Resuspend the beads in 500 µL of 65°C prewarmed SureSelect Wash 2 and mix on a vortex mixer for 5 seconds to resuspend the beads.
- **b** Incubate the samples for 10 minutes at 65°C in a recirculating water bath, heat block or equivalent. Occasionally mix on a vortex mixer.

  Do not use a tissue incubator. It cannot properly maintain temperature.
- **c** Briefly spin in a centrifuge.
- **d** Separate the beads and buffer on a magnetic separator and remove the supernatant.
- **e** Repeat step a through step d for a total of 3 washes.

  Make sure all of the wash buffer has been removed.

11 Mix the beads in 50 µL of SureSelect Elution Buffer on a vortex mixer for 5 seconds to resuspend the beads.

12 Incubate the samples for 10 minutes at room temperature. Occasionally mix on a vortex mixer.

13 Briefly spin in a centrifuge.

14 Separate the beads and buffer on a magnetic separator.

15 Use a pipette to transfer the supernatant to a new 1.5-mL microfuge tube.

The supernatant contains the captured DNA. The beads can now be discarded.

16 Add 50 µL of SureSelect Neutralization Buffer to the captured DNA.

17 Briefly mix on a vortex mixer.

Step 4. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 180 µL of homogenous AMPure XP beads to a 1.5-mL LoBind tube, and add 100 µL of captured DNA library. Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 0.5 mL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 step once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol completely evaporates.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at -20°C.

4 Addition of Index Tags by Post-Hybridization Amplification

Step 1. Amplify the captured library to add index tags 52
Step 2. Purify the sample using Agencourt AMPure XP beads 56
Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay 57
Step 4. Assess the quantity of each index-tagged library by QPCR 59
Step 5. Pool samples for Multiplexed Sequencing 60
Step 6. Prepare sample for cluster amplification 62

This chapter describes the steps to add index tags by amplification, purify, assess quality and quantity of the libraries, dilute the sample appropriately for cluster amplification, and pool indexed samples for multiplexed sequencing.

Step 1. Amplify the captured library to add index tags

Use reagents from:

- Herculase II Fusion DNA Polymerase (Agilent)
- SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2
- SureSelect Library Prep Kit, ILM

CAUTION Do not use amplification enzymes other than Herculase II Fusion DNA Polymerase. Other enzymes have not been validated.

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

Prepare 1 amplification reaction for each hybrid capture. Include a negative no-template control.

To see the nucleotide sequence in each of the index included in SureSelect reagent kits, see "SureSelect^{XT} Indexes for Illumina" on page 70.

1 For 1 library:
   - In a PCR tube, strip tube, or plate, prepare the reaction mix in Table 22, on ice. Mix well by gently pipetting up and down.

2 For multiple libraries:
   - **a** Prepare the reaction mix in Table 22, on ice. Mix well on a vortex mixer.
   - **b** Add 35 µL of the reaction mix to each well or tube.
   - **c** Add 1 µL of the appropriate index PCR Primer Index 1 through Index 16 (clear caps) from the SureSelect Library Prep Kit, ILM to each well and mix by pipetting.

     Use a different index primer for each sample to be sequenced in the same lane. Use Table 23 as a guide to determine the number of indexes to pool per sequencing lane.
   - **d** Use a pipette to add 14 µL of each DNA sample to each well or tube. Mix by pipetting. Change pipette tips between samples to avoid cross-contamination.

Table 22 Herculase II Master Mix

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Captured DNA	14 µL	
Nuclease-free water	22.5 µL	281.25 µL
5X Herculase II Rxn Buffer (clear cap) *	10 µL	125 µL
100 mM dNTP Mix (green cap)*	0.5 µL	6.25 µL
Herculase II Fusion DNA Polymerase (red cap)*	1 µL	12.5 µL
SureSelect ILM Indexing Post Capture Forward PCR Primer (orange cap)†	1 µL	12.5 µL
PCR Primer Index 1 through Index 16 (clear caps)‡	1 µL	
Total	**50 µL**	**437.5 µL (35 µL/reaction)**

* Included in the Herculase II Fusion DNA Polymerase (Agilent). *Do not use the buffer or dNTP mix from any other kit.*

† Included in the SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2.

‡ Use one of the 16 primers included in the SureSelect Library Prep Kit, ILM.

Table 23 Sequencing data requirement guidelines

Capture size	Optimal sequencing output per index
1 kb up to 0.5 Mb	0.1 to 50 Mb*
0.5 Mb up to 2.9 Mb	50 to 290 Mb*
3 Mb up to 5.9 Mb	300 to 590 Mb*
6 Mb up to 11.9 Mb	600 to 1190 Mb*
12 Mb up to 24 Mb	1.2 to 2.4 Gb*
Human All Exon v4	4 Gb
Human All Exon v4 + UTRs	6 Gb
Human All Exon 50 Mb	5 Gb
Human DNA Kinome	320 Mb
Mouse All Exon	5 Gb

* For custom libraries, Agilent recommends analyzing 100× amount of sequencing data compared to the Capture Library size for each sample. Pool samples according to your expected sequencing output.

3 Put the tubes in a thermal cycler and run the program in Table 24.

Table 24 PCR program

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	98°C	30 seconds
Step 3	57°C	30 seconds
Step 4	72°C	1 minute
Step 5		• Repeat Step 2 through Step 4 for a total of 12 to 16 times.
Step 6	72°C	10 minutes
Step 7	4°C	Hold

As with the pre-capture PCR amplification, minimize the number of PCR cycles used to enrich the captured DNA. The use of only half of the captured DNA for amplification lets you adjust the number of cycles by repeating the PCR if needed.

As an alternative, you can prepare one PCR master mix as outlined in Table 22. Split this master mix into three small-scale 10 µL PCR reactions and cycle for 10, 12, 14 or 16 cycles. Clean these PCR reactions using the AMPure XP protocol outlined in "Step 2. Purify the sample using Agencourt AMPure XP beads" on page 22 with these modifications: use 30 µL of AMPure XP beads and elute with 20 µL of nuclease-free water. Run these cleaned samples on a DNA 1000 chip on the Bioanalyzer, as described in "Step 3. Assess quality with the 2100 Bioanalyzer" on page 23.

Use the optimal cycle number to repeat PCR at the 50 µL reaction scale. See Table 25 for approximate number of cycles for a given library size. Results may vary based on library content.

Table 25 Cycle times

Capture Size	Cycles
1 kb up to 0.5 Mb	16 cycles
0.5 Mb up to 1.49 Mb	14 cycles
> 1.5 Mb	12 cycles
All Exon	10 to 12 cycles

Step 2. Purify the sample using Agencourt AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Mix the reagent well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
3. Add 90 µL of homogenous AMPure beads to a 1.5-mL LoBind tube, and add amplified library (~50 µL). Mix well on a vortex mixer and incubate for 5 minutes.
4. Put the tube in the magnetic stand. Wait for the solution to clear (approximately 3 to 5 minutes).
5. Keep the tube in the magnetic stand. Do not touch the beads while you carefully discard the cleared solution from the tubes.
6. Continue to keep the tube in the magnetic stand while you dispense 500 µL of 70% ethanol in each tube.

   Use fresh 70% ethanol for optimal result.
7. Let the tube sit for 1 minute to allow any disturbed beads to settle, and remove the ethanol.
8. Repeat step 6 and step 7 once.
9. Dry the samples on the 37°C heat block for 5 minutes or until the residual ethanol is completely evaporated.

   Do not dry the bead pellet to the point that the bead pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.
10. Add 30 µL nuclease-free water, mix well on a vortex mixer, and incubate for 2 minutes at room temperature.
11. Put the tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
12. Remove the supernatant (~30 µL) to a fresh 1.5-mL LoBind tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the samples at 4°C for up to a week, or at -20°C for longer periods.

Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay

NOTE

As an alternative, you can use the High Sensitivity D1K ScreenTape (Agilent p/n 5067-5363) and High Sensitivity D1K Reagents (Agilent p/n 5067-5364). For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

Use a Bioanalyzer High Sensitivity DNA Assay to assess the quality and size range. You may need to dilute your sample accordingly. Refer to the *Agilent High Sensitivity DNA Kit Guide* at http://www.chem.agilent.com/en-US/Search/Library/_layouts/Agilent/PublicationSummary.aspx?whid=59504.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the Agilent 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the appropriate assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify the results.

   Determine the concentration of the sample by integration under the peak.

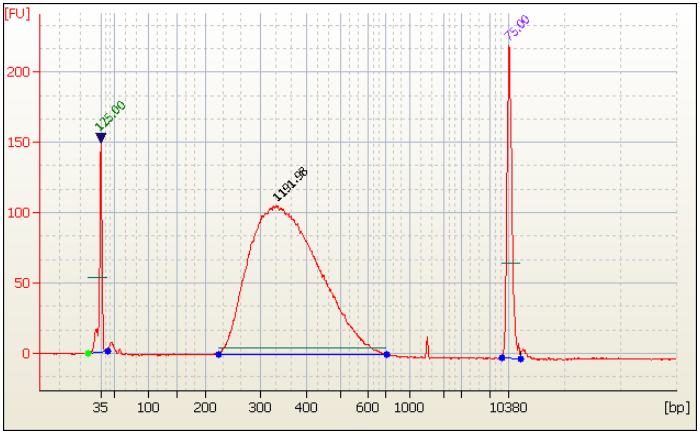

Figure 8 Analysis of Amplified Capture DNA using the High Sensitivity DNA Kit. The electropherogram shows a peak in the size range of approximately 300 to 400 nucleotides.

Step 4. Assess the quantity of each index-tagged library by QPCR

Refer to the protocol that is included with the QPCR NGS Library Quantification Kit (Illumina GA) for more details to do this step.

1. Use the QPCR NGS Library Quantification Kit (Illumina GA) to determine the concentration of each index-tagged captured library.
2. Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.
3. Dilute each index-tagged captured library such that it falls within the range of the standard curve.

   Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.
4. Prepare the QPCR master mix with Illumina adaptor-specific PCR primers according to instructions provided in the kit.
5. Add an aliquot of the master mix to PCR tubes and add template.
6. On a QPCR system, such as the MX3005P, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.
7. Use the standard curve to determine the concentration of each unknown index-tagged library, in nM.

The concentration will be used to accurately pool samples for multiplexed sequencing.

Step 5. Pool samples for Multiplexed Sequencing

1 Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool. For each library, use the formula below to determine the amount of index sample to use.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)} \text{ where}$$

V(f) is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool, for example, 10 nM for the standard Illumina protocol

is the number of index, and

C(i) is the initial concentration of each index sample.

Table 26 shows an example of the amount of 4 index-tagged (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

Table 26 Example of index volume calculation for a total volume of 20 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

3 If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

4 Proceed to template denaturation and flow cell preparation. Refer to the appropriate Illumina protocol.

Exact library pool dilution and processing can vary based on the flow cell capacity and analysis pipeline versions being used. Refer to the appropriate Illumina user guide for instructions. This protocol has been validated with 36-base paired-end reads. However, read length can be adjusted to achieve the desired research goals.

Step 6. Prepare sample for cluster amplification

In this step you set up cluster amplification.

Conditions are optimized to provide 700K to 900K clusters/mm^2 on the GAIIx and 400K to 600K clusters/mm^2 on a HiSeq instrument.

CAUTION Pooled captures from SureSelect^{XT} libraries must be sequenced according to the Illumina Multiplexed Sequencing protocol. Samples with a single index tag can be sequenced with the Paired-end protocol, but make sure you use reagents for multiplexed sequencing.

Genome Analyzer IIx

Use reagents from the TruSeq Cluster Generation Kit appropriate for your instrument:

- HT1 (Hybridization Buffer)
- HP3 (2 N NaOH)

1. Dilute 30 fmol (3µL) of the 10 nM multiplexed sample pool with 16 µL of Buffer EB (10mM Tris-Cl, ph 8.5) for a total volume of 19 µL.
2. Add 1 µL of HP3 (2 N NaOH).
3. Mix the sample briefly on a vortex mixer and pulse centrifuge.
4. Incubate for 5 minutes at room temperature to denature the DNA.
5. Place the sample on ice until you are ready to proceed to final dilution.
6. Dilute 8 µL of denatured DNA with 992 µL of pre-chilled HT1 (Hybridization Buffer) for a final concentration of 12 pM.
7. Mix the sample briefly on a vortex mixer and pulse centrifuge.
8. Continue with cluster generation. Use the TruSeq SBS Kit v5–GA (36-cycle) and the Illumina multiplexed sequencing protocol.

HiSeq2000 with PhiX spike-in controls

Use reagents from the appropriate for your instrument:

- HT1 (Hybridization Buffer)
- HP3 (2 N NaOH)

Use the PhiX Control Kit V2 (Illumina CT-901-2001) for:

- PhiX Control

1. Prepare a 1:20 dilution of HP3 (2 N NaOH) down to 0.1N NaOH.
2. Prepare 10 nM (10 fmol/µL) dilutions of the amplified capture, based on the Bioanalyzer quantitation.
3. Add 20 fmol (2 µL) of the 10 nM multiplexed sample pool into 8 µL of Buffer EB (10mM Tris-Cl, ph 8.5) to make a 2 nM solution.
4. Add 10 µL of 0.1 N NaOH.
5. Mix the sample briefly on a vortex mixer and pulse centrifuge.
6. Incubate for 5 minutes at room temperature to denature the DNA.
7. Add 980 µL of HT1 (Hybridization Buffer) to the denatured DNA to make 20 pM template solution.
8. Mix the sample briefly on a vortex mixer and pulse centrifuge.
9. Prepare 4 pM template by mixing 200 µL of 20 pM solution with 800 µL of Pre-Chilled HT1 (Hybridization Buffer).

   If densities higher than 400K-600K clusters/mm^2 are desired, prepare a more concentrated sample from the 20 pM solution.
10. Mix the sample briefly on a vortex mixer and pulse centrifuge.
11. Remove 10 µL from solution (1000 µL) to get 990 µL.
12. Add 10 µL of PhiX Control.
13. Mix the sample briefly on a vortex mixer and pulse centrifuge.
14. Dispense 120 µL of diluted denatured sample DNA template and PhiX Control into a strip tube.
15. Place on ice until ready to use.
16. Continue with cluster generation. Use TruSeq SBS Kit–HS (50 cycle) and the Illumina multiplexed sequencing protocol.

CAUTION For Human All Exon captures, to get optimal performance and specificity, do not sequence using longer reads, such as 2x100 bases. The SureSelect Human All Exon kits and protocol were optimized for 2x76 base sequencing.

5 Reference

SureSelect Reagent Kit Content 66
Other Reagent Kits Content 68
SureSelect^{XT} Indexes for Illumina 70
Alternative Capture Equipment Combinations 71

This chapter contains reference information.

SureSelect Reagent Kit Content

Each SureSelect Reagent Kit contains one or more of each of these individual kits:

Table 27 SureSelect Reagent Kit Contents

Product	Storage Condition	16 Reactions	96 Reactions	480 Reactions
SureSelect Target Enrichment Kit Box #1	Room Temperature	5190-4393	5190-4394	5190-4395
SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2	-20°C	5190-4455	5190-4456	5190-4457
SureSelect Library Prep Kit, ILM	-20°C	5500-0105	5500-0075	

The content of each of these kits are described in the next tables.

Table 28 SureSelect Target Enrichment Kit Box #1

Kit Component
SureSelect Hyb #1 (orange cap, or bottle)
SureSelect Hyb #2 (red cap)
SureSelect Hyb #4 (black cap, or bottle)
SureSelect Binding Buffer
SureSelect Wash 1
SureSelect Wash 2
SureSelect Elution Buffer
SureSelect Neutralization Buffer

Table 29 SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box #2

Kit Component
SureSelect Hyb #3 (yellow cap)
SureSelect Indexing Block #1 (green cap)
SureSelect Block #2 (blue cap)
SureSelect Indexing Block #3 (brown cap)
SureSelect RNase Block (purple cap)
SureSelect ILM Indexing Pre Capture PCR Reverse Primer (clear cap)
SureSelect ILM Indexing Post Capture Forward PCR Primer (orange cap)

Table 30 SureSelect Library Prep Kit, ILM

Kit Component
10X End Repair Buffer (clear cap)
T4 Polynucleotide Kinase (orange cap)
10X Klenow Polymerase Buffer (blue cap)
T4 DNA Ligase (red cap)
Exo(-) Klenow (red cap)
T4 DNA Polymerase (purple cap)
Klenow DNA Polymerase (yellow cap)
dATP (green cap)
dNTP Mix (green cap)
SureSelect Adaptor Oligo Mix (brown cap)
SureSelect Primer (brown cap)
PCR Primer Index 1 through Index 16 (clear caps)
5X T4 DNA Ligase Buffer

Other Reagent Kits Content

These reagents are from kits other than the SureSelect Reagent kit. Make sure you use only the reagents listed here.

Table 31 Herculase II Fusion DNA Polymerase (Agilent)

Component
DMSO (green cap)
5X Herculase II Rxn Buffer (clear cap)
100 mM dNTP Mix (green cap)
Herculase II Fusion DNA Polymerase (red cap)

Table 32 D1K Reagents (Agilent p/n 5067-5362)

Components
D1K ladder
D1K sample buffer

Table 33 High Sensitivity D1K Reagents (Agilent p/n 5067-5364)

Components
High-Sensitivity D1K ladder
High-Sensitivity D1K sample buffer

Table 34 TruSeq Cluster Generation Kit*

Components
HT1 (Hybridization Buffer)
HP3 (2 N NaOH)

* Use the Illumina Cluster Generation Kit that is appropriate for your instrument and setup. See Table 5 on page 11.

Table 35 PhiX Control Kit V2 (Illumina CT-901-2001)

Components
PhiX Control

SureSelect^{XT} Indexes for Illumina

The nucleotide sequence of each of the SureSelect^{XT} index is listed in Table 36.

Table 36 SureSelect^{XT} Indexes 1-16

Index Number	Sequence
1	ATCACG
2	CGATGT
3	TTAGGC
4	TGACCA
5	ACAGTG
6	GCCAAT
7	CAGATC
8	ACTTGA
9	GATCAG
10	TAGCTT
11	GGCTAC
12	CTTGTA
13	AAACAT
14	CAAAAG
15	GAAACC
16	AAAGCA

Alternative Capture Equipment Combinations

Table 37 lists combinations of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape) other than those used in this protocol that have shown minimal evaporation.

Refer to this list for additional of equipment combination options for hybridization. Note that minimal evaporation is needed to ensure good capture results.

Table 37 Tested options that show minimal evaporation

PCR Machine	Plate/Strips	Cover	Comments
Agilent Mx3005P QPCR	Mx3000P Strip Tubes (401428)	MX3000P Optical Strip Caps (401425)	Heated Lid
Agilent Mx3005P QPCR	MicroAmp Optical 96-well reaction plate (N801-0560)	MicroAmp Clear Adhesive Film (4306311)	Heated Lid; ABI compression pad (4312639) Use two layers of film.
ABI GeneAmp 9700	MicroAmp Optical 96-well Reaction Plate (N801-0560)	MicroAmp Caps (8caps/strip) (N801-0535)	Heated Lid
ABI Veriti (4375786)	MicroAmp Optical 96-well Reaction Plate (N801-0560)	MicroAmp Clear Adhesive Film (4306311)	Heated Lid; ABI compression pad (4312639) Use two layers of film.
Eppendorf Mastercycler	Eppendorf 8-Tube PCR Tubes	Attached lids	Lid heating set to 75°C
BioRad (MJ Research) PTC-200	Agilent strip tubes 410022 (Mx4000)	Agilent Optical cap 410024 (Mx4000)	Heated Lid
BioRad (MJ Research) PTC-200	Agilent strip tubes 410022 (Mx4000)	Agilent Optical cap 401425 (Mx3000/3005)	Heated Lid
BioRad (MJ Research) PTC-200	Agilent 96-well Plate 410088 (Mx3000/3005)	Agilent Optical cap 401425 (Mx3000/3005)	Heated Lid

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In This Book

This guide contains information to run the SureSelect^{XT} Target Enrichment System for Illumina Paired-End Sequencing protocol with the Illumina HiSeq and MiSeq Multiplexed Sequencing Platforms.

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